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HEMOLYTIC ACTIVITY OF ANIMAL VENOMS. I. CLASSIFICATION IN DIFFERENT TYPES AND ACTIVITIES (*)

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According to Fontana (9) the first remarks that snake venoms are hemolytic were due to Mead in 1739, contrary to his own observations, following those of James and Backer in the same century. In the nineteenth century, in 1884, Lacerda (19) had noted that red blood corpuscles agglutinated, became crenated and were hemolyzed by the action of venom. Weir-Mitchell and Reichert (21) also described, in 1886, transformations of erythrocytes when venom was added to blood "in vitro", remarking that they agglutinated and became spherical.

In 1889 Stephens (32) observed an increase of the hemolytic activity of Cobra venom by the addition of serum, but he did not give an explanation for this fact. Some years later, Flexner and Noguchi (8) noticed that some venoms did not hemolyze washed red cells, but if small amount of serum was added to them the venom hemolytic activity appeared. They considered the serum as a complement. This work started a new era in the study of the hemolytic activity of venoms. In 1902 Calmette (4) investigating the complement activity of serum on the hemolytic activity of snake venoms observed that its role was not similar to that of alexines since heating at 62.°C did not destroy it; on the contrary, the activity was increased and maintained even at 80°C. A further step was made in 1902 and 1903 by Kyes (16,17) who, working in the laboratory of Ehrlich, demonstrated that the serum thermostable substance, a necessary intermediate for hemolysis, could be extracted by alcohol, and among the serum alcohol soluble substances, only lecithin had this activity. Kyes prepared a complex ("schlangengiftlecithid") with lecithin and Cobra or *Crotalus adamanteus* venom, which hemolyzed blood instantly. Thus the activating substance of venom-hemolysis was defined for the first time.

A series of works followed this definition and among them that of Delezenne and Ledebt in 1911 (6) should be noted as it demonstrated that by a

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catalytic action, the venom liberates from serum a substance with hemolytic activity. This conclusion was reached by studying the action of *Naja naja* venom on horse serum in the hemolysis phenomenon. Later, in 1914, Delezenne and Fourneau (5), having performed a chemical study on the hemolytic compound, called it "lisolecithin" and defined in the chemical point of view the venom provoked hemolysis reaction as being a saponification.

Lamb in 1905 (20), having studied the hemolytic activity of different snake venoms on the same species of erythrocytes, divided them in two groups: one group which hemolyzed dog erythrocytes directly, and another having only indirect action, *i.e.*, requiring the presence of serum or lecithin. He included in the first group venoms of *Naja naja* (*tripudians*) and *Vipera russellii*. Direct hemolytic action of *Naja naja* venom was also found by Slotta and Borchert in 1954 (31).

In 1904, Noc (25) determined the hemolytic activity of different snake venoms on horse red cells. He concluded that venoms classification according to their hemolytic intensity was correlated to the classification of species. However Pestana in 1908 (27) after observations on the hemolytic action of Brazilian snake venoms, considered this activity independent from the zoological classification, because though having found activity variations from one species to another, these variations could also be found in venoms of the same species.

Noc suggested in 1904 (25) that the hemolysins of different snake species were one substance or if more than one they were of very similar nature as they could be neutralized by antivenom serum prepared with venom of any species. For example anti-Cobra serum neutralized the hemolysins of *Naja niger*, *Bungarus*, *Bothrops jararaca*, *B. urutu* and others. It was also Pestana in 1916 (28) who considered the venom hemolytic substance of some Brazilian snakes as one and the same substance. He verified that the anticrotalic serum could neutralize the venom of *Crotalus durissus terrificus* and also those of *B. atrox* and *B. lanceolatus*. The same was found for the antibothropic serum that prevented the hemolytic activity of *C. d. terrificus* venom. However Houssay and Negrete in 1922 (13) had reached an opposite conclusion, *i.e.*, that the hemolysins were not alike in the different venoms, not even among those of southamerican snakes.

It had been stated that the venoms toxic activity was directly related to the hemolytic activity since antivenom sera in neutralizing hemolysins also destroyed the toxin (Stephens and Myers, 1898) (33). This suggested the utilization of the method for sera titrations (Calmette, 1902 and Noc, 1904) (4, 25). On the other hand, Pestana (28) had already mentioned a dissociation between hemolytic and toxic substance for antierotalic serum that did not neutralize the toxicity of *Bothrops* venoms, though preventing the hemolytic activity of *B. atrox* and *B. lanceolatus* venoms besides that of the *Crotalus* one, specific to it. This distinction between these two activities was confirmed in 1958 by Ohsaka (26) who separated

electrophoretically at least two independent hemolytic fractions from the toxic one in the venom of *Trimeresurus flavoviridis*, he also found (26) that more than one hemolytic substance existed in the same venom. It must be mentioned that some years before (1952-3), Neumann and Habermann (23) and Neumann, Habermann and Hansen (24) had already isolated two different hemolytic fractions, one having direct activity and the other an indirect one (23,24). The existence of two of these fractions was confirmed in 1954 by Slotta and Borchert (31) who, however attributed direct hemolytic activity to both.

Morgenroth and Carpi (22) pointed out in 1906 that bee venom (*Apis mellifica*) was hemolytic, a finding confirmed by many research workers, including Essex, Markowitz and Mann in 1930 in 1930 (7); but thes eauthors found reason to believe in the similarity of this venom with that of the rattlesnake, based on the hemolytic activity and others.

Another remarkable fact that should be pointed out in relation to venom-provoked hemolysis is that the hemolytic activity is not always proportional to the venom concentration, as was already demonstrated by Stephens and Myers in 1898 (33), who verified decreasing hemolysis by greater concentrations of Cobra venom. Houssay and Negrete in 1922 (13) made the same statement in relation to different venoms.

The purpose of this paper was to study the relative potency of the hemolytic activity of different animal venoms with a more exact quantitative method than those utilized till now, and to veirfy, systematically, the eventual existence of direct hemolytic activity and the venom capacity of transforming hemoglobin to methemoglobin.

MATERIAL AND METHODS

Saline solution — All solutions and suspensions usend in hemolysis tests were made in a saline solution which contained 0,85% NaCl (w/v) and was 0,15 M in relation to $CaCl_2$, and 0,5 M as regards $Mg\ Cl_2$. Only the washing of erythrocytes was made in 0,85% NaCl (w/v).

Venom — The experiments were performed with dried venoms of the Instituto Butantan stock. The solutions in saline were made just before use. *Bothrops jararaca standard venom* — This was chosen as standard since it is available in greater amounts. Each time that a series of tests had to be performed a fresh solution of *B. jararaca* venom was prepared and used as standard.

Serum — Serum used in comparative hemolysis of different venoms was obtained from horse blood after three hours standing at room temperature. After centrifugation and separation it was kept at — 15°C until used.

For the obtention of hemolysis curves the serum was inactivated at 55°C during 30 minutes before being stored in the freezer. Heat treated serum proved to be more stable than fresh serum.

Erythrocytes — Sheep blood was collected on an equal volume of Alsever solution and kept at 5°C up to the time of use. Prior to the hemolysis tests the red blood cells were washed by centrifugation four times with 0,85% (w/v) NaCl and then suspended in the saline solution in such a way as to contain 100 million erythrocytes per ml. A standard curve was prepared for the resulting erythrocyte suspension. For this purpose, different amounts (up to one ml) of the erythrocyte suspension were made up to 5 ml with distilled water. The hemolyzed mixture was centrifuged and the optical absorbance of the supernatant was determined at 540 mμ. The resulting standard curve, drawn by plotting the optical absorbance against the number of lyzed cells, was used to determine the intensity of hemolysis produced by the venoms with which the standardized suspension was incubated.

The wave length of 540 mμ was fixed for absorption determinations because this zone corresponds to the smallest absorption by serum colour (bilirubin) as well as by the brown colour (methemoglobin) resulted by the action of almost all venoms.

Test for indirect hemolysis — The reaction mixtures, prepared by adding 1 ml of standardized erythrocyte suspension, 1 ml of horse serum, 2 ml of the saline solution and 1 ml of saline containing 5 mg of venom, were kept at 37°C for one hour. The tubes containing the different mixtures were then transfered to an ice-water bath and centrifuged. The degree of hemolysis was calculated from the observed absorbance at 540 mμ by means of the standard curve prepared as described. Since erythrocyte suspensions prepared on different days could present fluctuations on sensitivity to hemolysis, and this variation should affect the calculated intensity of hemolysis, all hemolysis tests included a determination of the intensity of hemolysis produced by a constant amount (5 mg) of standard venom of *Bothrops jararaca*. Thus, the data for the different venoms could be expressed in terms of the hemolytic index (HI) defined by the equation

$$HI = \frac{N_x}{N_j}$$

in which N_x and N_j are the number of erythrocytes lyzed (calculated from the observed absorbance) by 5 mg of the tested venom and by the same amount of *B. jararaca*, respectively. Since the same venom of *B. jararaca* was used throughout this work, the HI figure of the different venoms studied in these experiments should permit a more accurate comparison of their relative hemolytic potencies.

As the same venom hemolyzed different number of erythrocytes in different experiments, one of the values obtained with *B. jararaca* venom was considered as standard. In all experiments a determination was done with the standard venom and the result was corrected in function of the value considered as a parameter. This compensation gave a correction factor to the sensibility or resistance varia-

tions of red cells during the experiments, and this factor was used in correcting all values. This way a more exact and more direct comparison could be made among the relative potencies, and the hemolytic index of each venom gained more equilibrium.

Hemolysis curves were obtained from different venom concentrations acting on a constant number of erythrocytes.

Direct hemolysis — The same technique was used for these determinations as that used for indirect hemolysis, except that horse serum was omitted.

RESULTS

Almost all of the experimented snake venoms used (table 1) demonstrated indirect hemolytic activity, being exceptions only the venoms of *B. eotiara, M. frontalis* and a white venom of *B. jararaca.* In this group are the following *Bothrops* venoms: *alternatus*, young *jararaca, insularis, atrox* of Marajó island, *fonsecai, atrox, neuwiedi, atrox asper, itapetiningae* and *pradoi.* Exceptions were the venoms of *B. jararaeussu* which had only half of the potency of other *Bothrops* and the venom of *B. eotiara* practically nonhemolytic.

Venoms of *Vipera* genus like *V. russellii, lebetina, amodytes moutaudoni* were also very hemolytic as well as other Indian venoms like those of *Naja naja, Bungarus caeruleus* and *Ecchis carinatus.* The venom of *Trimeresurus flavoviridis* demonstrated a potency similar to that of *Bothrops.*

Venoms of *Crotalus* genus demonstrated weak hemolytic activity as it is indicated by the index of yellow and white venoms of *C. d. terrificus* and that of *C. d. terrificus* of Marajó island (Brazil). Venom of *C. durissus durissus* (Costa Rica) was an exception and so was that of *C. d. terrificus* from the state of Ceará (Brazil) since both were very active regarding their hemolytic activity. Included in the group of weak venoms is also that of *A. piscivorus.*

The other animal venoms studied *Tityus bahiensis, T. serrulatus, Lyeosa erythrognata, Phoneutria fera,* did not show hemolytic activity, only that of *Bufo marinus* acted slightly on red blood cells. However, the venom of *Apis mellifica* was two times stronger than the standard venom, being the most hemolytic among the venoms studied.

Hemolytic venoms changed also hemoglobin to methemoglobin easily recognized by the brown colour produced on the hemoglobin solutions and red blood cells, excepting the white hemolytic venoms of *A. mellifica, B. itapetiningae* and the white venoms of *C. d. terrificus* and *B. jararaca.* Non hemolytic venoms did not change the colour of erythrocytes, excluding those from *M. frontalis* and *B. eotiara* (table 1); but these venoms were yellow. Therefore, there was a correlation between the venom colour and methemoglobin production. Yellow venoms, whether hemolytic or not, formed methemoglobin and the reciprocal has also been stated: white venoms, hemolytic or not, did not form methemoglobin.

TABLE 1 — Indirect hemolytic activity of different venoms (only *Apis mellifica* venom had also direct activity)

Hemolytic indexes and capacity of producing methemoglobin in relation to sheep erythrocytes and horse serum

VENOMS	Erythrocytes x 10^6 hemolyzed by 5 mg of venom with correction	Hemolytic indexes III	Methemoglobin formation
Apis mellifica	141	2,20	absence
Naja naja	82	1,28	+
Vipera lebetina	81	1,26	+
Bungarus caeruleus	78	1,22	+
Vipera ammodytes montandoni	75	1,17	+
Vipera russellii	73	1,14	+
Bothrops alternatus	72	1,12	+
Bothrops jararaca (young snake)	72	1,12	+
Bothrops insularis	68	1,06	+
Crotalus durissus durissus	68	1,06	+
Lachesis muta muta	68	1,06	+
Bothrops atrox (Marajó island)	67	1,04	+
Bothrops fonsecai	67	1,04	+
Bothrops jararaca (*standard*)	*64*	*1,00*	+
Bothrops atrox	62	0,97	+
Bothrops neuwiedi	62	0,97	+
Ecchis carinatus	61	0,95	+
Bothrops atrox asper	60	0,93	+
Bothrops itapetiningae	59	0,92	absence
Bothrops pradoi	58	0,91	+
Crotalus durissus terrificus (Ceará)	57	0,89	+
Trimeresurus flavoviridis	52	0,81	+
Bothrops jararacussu	33	0,52	+
Agkistrodon piscivorus	29	0,45	+
Crotalus durissus terrificus (yellow venom)	29	0,45	+
Crotalus durissus terrificus (white venom)	29	0,45	absence
Bufo marinus	10	0,16	+
Crotalus durissus terrificus (Marajó island)	7	0,11	+
Bothrops cotiara	2	0,03	+
Micrurus frontalis	no hemolysis	no hemolysis	+
Tityus bahiensis	" "	" "	absence
Tityus serrulatus	" "	" "	absence
Androctonus australis	" "	" "	absence
Lycosa erythrognata	" "	" "	absence
Phoneutria fera	" "	" "	absence
Loxosceles rufipes	" "	" "	absence
B. jararaca (white venom)	" "	" "	absence

"Direct hemolytic activity" could only be found in *Apis mellifica* venom. All other venoms acted only indirectly.

Different concentrations of one venom, up to 20 mg, acting on constant number of erythrocytes, 100 x 10^6, had shown that hemolysis was not always proportional to venom concentrations. To an increase of venom concentration there was not always a corresponding increase in hemolysis (fig. 1). This fact lead to choose the concentration of 5 mg for index determinations, since this zone still corresponded to an intense hemolytic activity of almost all venoms;

greater concentrations showed frequently an inhibition and weaker solutions had already decreasing activity.

For the same reason, venoms were divided not according to the snake species anymore, but according to the curve type of hemolysis obtained, what permitted to classify the venoms according to 6 types of hemolysis curves:

Type I — curve given by venoms of *Agkistrodon piscivorus* (fig. 1, curve 1) and of *Naja naja*, demonstrating a linear function.

Type II — a sigmoidal curve obtained with venoms of *B. insularis* (fig. 1, curve 2) *B. jararaca, B. alternata, B. pradoi, V. bebetina, C. d. durissus, C. d. terrifeius* of Marajó island and *Eechis carinatus*.

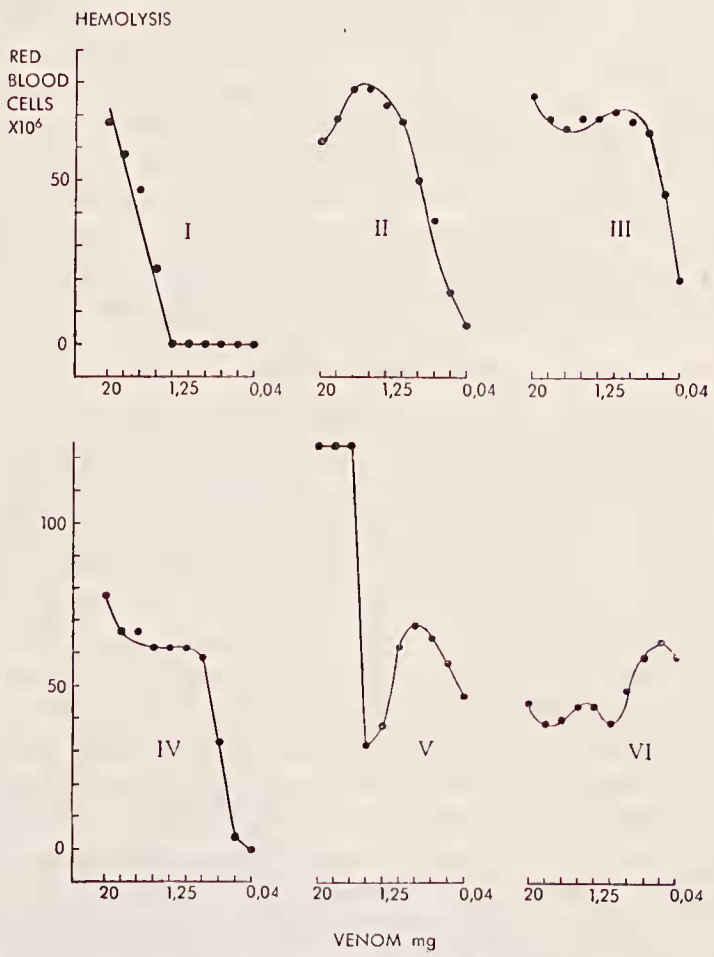

FIG. 1 — Types of curves of hemolytic activity of the venoms dilutions. (Sheep red blood cells and horse serum.)
I — *Agkistrodon piscivorus*. II — *Bothrops insularis*. III — *Bothrops atrox*. IV — *Bothrops atrox asper*. V — *Apis mellifica*. VI — *Trimeresurus flavoviridis*.

Type III — obtained with venoms of *B. atrox* (fig. 1, curve 3), *B. atrox* of Marajó island, *B. neuwiedi*, *B. fonsecai*, *B. jararacussu*, *L. muta* and *V. russellii*.

Type IV — showed by venoms of *B. atrox asper* (fig. 1, curve 4) and white venom of *C. d. terrificus*.

Type V — curve obtained with venom of *A. mellifica* (fig. 1, curve 5).

Type VI — curve obtained with venom of *Trimeresurus flavoviridis* (fig. 1, curve 6).

Hemolysis curves of venoms of *B. pradoi*, *C. d. terrificus* from Marajó island, *E. carinatus*, *N. naja* and *V. lebetina* were constructed up to 5 mg, as only small amounts of those venoms were available.

Venom of *A. mellifica* as well as that of standard (*B. jararaca*) were submitted to heating at 100°C for 10 minutes. This treatment caused a heavy precipitation of the venom of *B. jararaca* with disappearence of hemolytic activity, while the venom of *A. mellifica* remained active, showing however a decrease in poteney. In presence of 1 N NaOH the hemolytic activity of this venom is destroyed by heating at 100° C for 10 minutes, while, in presence of 1 N HCl, heating causes only a change in the characteristic curve of hemolysis. The hemolytic activity of *Apis mellifica* venom was not modified by the addition of NaOH 0.1 N or HCl 0.1 N after 15 minutes at room temperature.

DISCUSSION

DIRECT HEMOLYSIS — Excluding *Apis mellifica* venom, none of the experimented venoms had direct hemolytic activity, *i.e.*, they were not able to hemolyze washed red blood cells in absence of serum. This fact discords of Lamb's experiments (20), who had reported direct hemolylis with venoms of *Naja naja* (*N. tripudians*) and *Vipera russellii*, and of the observations of Slotta and Borchert (31) who had also attributed such activity to *Naja naja* venom.

This difference may be explained by considering the different type of erythrocytes used. While the lack of hemolysis reported in this paper was observed on sheep erythrocytes, Lamb used dog red cells and Slotta and Borchert had employed human erythrocytes, and many reports already exist about the difference of susceptibility of red blood cells of different animal species, as was first seen by Mitchell and Reichert (21) and systematically studied by Kelen, Rosenfeld and Nudel (15). One could also think that the authors had used badly preservated erythrocytes in their experiments, since we could observe that red cells kept at 4°C for many days increased their sensitivity to venom hemolysis. Another important factor involved is the kind of serum and its conservation (15).

The direct hemolytic activity of venom of *Apis mellifica* was observed besides its indirect activity, confirming the findings of Neumann and Habermann (23), Neumann, Habermann and Hansen (24) and Slotta and Borchert (31).

NON HEMOLYTIC VENOMS — Scorpion venoms like *Tityus serrulatus*, *Tityus bahiensis* and *Androctonus australis* did not show hemolytic activity confirming data of Balozet (1) who stated that scorpions of *Buthidae* family did not have hemolytic venom, and to this family belong the *Androctonus australis* and the *Buthus occitanus* that he has observed, as well as the *Leiurus quinquestriatus* and the *Tityus bahiensis* studied by Houssay (12).

Therefore it seems that it may be assumed that scorpion venoms of the genus *Tityus*, *Androctonus*, *Buthus* and *Leiurus*, all of them belonging to the *Buthidae* family, do not have hemolytic activity. This conclusion, however, does not apply to the genus *Buthacus* belonging to the same family and to the *Scorpionidae* family since Balozet (1) detected indirect hemolytic activity in venoms of *Scorpio maurus* and *Buthacus arenicola* (2), the latter having shown stronger activity than many snake venoms.

All snake venoms were hemolytic, excepting venoms of *Micrurus frontalis* and *Bothrops cotiara* that did not hemolyze sheep red blood cells. Brazil and Pestana (3) had refered a weak hemolytic activity of venom of *M. frontalis* on horse red cells.

Spider venoms, *Lycosa erythrognata*, *Phoneutria fera* and *Loxosceles rufipes* were another group that did not hemolyze sheep erythrocytes. Walbum in 1915 (35) and Houssay in 1916/17 (10,11) had already mentioned the absence of hemolytic activity of some spider venoms. They could find very active hemolysins only in the eggs of these animals or in body extracts.

It should be stressed that the concept of hemolytic capacity of venoms must be also defined in relation to species of erythrocytes used, since Kelen, Rosenfeld and Nudel (15) observed that venoms considered as non hemolytic for one species of cells were very active for another one, as well as a variation of hemolysis intensity of the same factors could also be stated.

RELATIVE POTENCIES — Since the experimented venoms showed an indirect hemolytic activity, their hemolytic potencies were calculated according to this activity. Table 1, containing all hemolytic indexes of venoms, demonstrates clearly the lack of direct relation between hemolytic activity and genus or even species of snake, confirming observations already refered by Pestana (27). Thus, venoms of *Bothrops* genus show all indexes HI above 0,90, however HI of *B. jararacussu* is only 0,52. In case of venoms of the same species, while those of *C. d. terrificus* are weakly hemolytic, HI = 0,45, venom of snakes of same species and genus, but from the state of Ceará (Brazil), was very active with HI = 0,89.

In some cases the order of hemolytic potency found (table 1) for the animal venoms studied in this work differs from classifications published previously (13, 18, 25, 27) which have been derived from studies made without a) quantitatively accurate technique of hemolytic tests, and b) a strict definition of the species, concentration and preservation of the erythrocytes used.

HEMOLYSIS CURVES — The first purpose of this paper was only to investigate the hemolytic potencies of animal venoms, but during the titrations it could be demonstrated with the simple method presented and through the different curve types obtained that venoms probably contain variable number of hemolytic substances.

Type I curve obtained may be considered as resulting from the action of one kind of hemolytic substances; it was called component A_1. Considering the mechanism of indirect hemolysis, *i.e.*, activation of serum lecithin by the venom and formation of lysocithin, A_1 would be the venom lecithinase.

Type II curve shows an inhibition of higher venom concentrations, and this inhibition has been already described in 1898 by Stephens and Myers (33) and in 1922 by Houssay and Negrete (13), though no one of them had indicated any explanation to this fact. A plausible hypothesis would be to assume that the venom contains another enzyme A_2 acting on the same substrate, lecithin, but splitting the molecule at a different point, so that inactive substances to hemolysis result; if A_2 lipase is less active then A_1, it can still act first in high concentrations on the common substrate, impeding or delaying the action of A_1. By dilution, the latter acts before A_2 once it is more active, and so hemolysis occurs. This mechanism would be analogue to that of blood coagulation by the action of coagulant and proteolytic venoms, as described by Rosenfeld, Hampe and Kelen (30); in their experiments the dilution curve of the coagulant activity of a venom having both components, shows that high concentrations do not coagulate plasma because, though the proteolytic substances are less active than the coagulant ones, they are in such an amount that it permits them to act first on the common substrate, fibrinogen, in hydrolysing it before the coagulant fraction could turn it to fibrin. This way fibrinogen molecule is parted in non coagulable fractions. As concentration decreases, proteolytic substances are not able to act anymore, and so transformation of fibrinogen in fibrin takes place under action of the coagulant substances.

Venoms of type III besides of having the hemolytic factor A_1 and the inhibitor A_2 like the type II, they have also a weak indirect hemolytic component, acting only in very high concentrations. This component was called A_3.

Venoms giving type IV curve have the same components as those of type III. However a difference is observed concerning the inhibitor concentration. Its presence is only evidenced in the curve by a "plateau" between the two indirect hemolytic actions.

Type V curve, characteristic to bee venom, evidences the presence of components A_1 and A_2 in higher concentrations than in the experimented snake venoms. Besides it has a hemolytic component also active in high venom concentrations like the A_3 with the difference however of being directly hemolytic. It does not require an intermediate substance to hemolyse the red cells. This component was called B.

In addition to the above mentioned components two others were evidenced in hemolysis curve of type VI obtained with *Trimeresurus flavoviridis* venom: components A_4, inhibitor, and A_5 indirectly hemolytic, both acting in high venom concentration.

VENOM CONCENTRATION — The importance of venom concentration in the determination of relative potencies is another factor demonstrated by the hemolysis curves since an activity degree due to different substances correspond to the action of different venom concentrations on a same number of erythrocytes.

Therefore the hemolytic index has just a relative value, once that the same concentration of different venoms acting on a constant number of red cells does not refer to identical activities. The index calculated in relation to one concentration will not always be comparative of the same effect. Thus, if to 5 mg concentration of some venoms corresponds the stage of lecithin activation for hemolysis of 100 million erythrocytes, to this same concentration of other venoms corresponds the stage where the inhibitory substances of serum are still activated. Therefore, there is not an absolute criterium in choosing venom concentrations in order to be able to compare their hemolytic potencies.

MULTIPLICITY OF HEMOLYTIC FACTOR — There almost do not exist data on the existence in venoms of more than one factor with hemolytic activity. In relation to bee venom, two kinds of hemolysins were identified, one as being direct and another indirect (23, 24, 31); in 1921 Houssay (14) had observed two components responsible for the hemolytic activity of *B. neuwiedi* (*Lachesis*) venom when trying to isolate from this venom its active substance. And more recently Ohsaka (26) separated electrophoretically two hemolytic fractions in Habu venom (*Trimeresurus flavoviridis*).

The hemolysis curves obtained demonstrate quite well the variable number of hemolytic substances existing in venoms, permitting to classify them according to their hemolytic activity and also to show that this activity is much more complex than the way it is regarded as.

The knowledge of the mechanism of hemolysis can be useful to understand what does occur in the chemical point of view in hemolysis resulting from other causes than the animal venom, since enzymes of the same kind may occur or may be activated in the organism of other animals.

The curves obtained with a simple dilution method and quantitative estimation of hemolytic activity may be useful in further researches on the subject by verifying the hemolytic components responsible for this venom activity, in the point of view of hemolysis as well as that of the venom itself.

INDEPENDENCE OF HEMOLYTIC ACTIVITY FROM OTHER VENOM ACTIVITIES — The lack of direct relation between venoms hemolytic activity and their

coagulant and proteolytic substances is well demonstrated if comparing the indexes refering all of them (table 2). This comes in favour of Pestana's conclusion in 1916 (28), who had dissociated hemolytic and toxic substances of some venoms, in contradiction to conclusions of Stephens and Myers (33), Calmette (4) and Noe (25). Ohsaka (26) in 1958 has also separated hemolytic substances from the toxic one of Habu venom (*T. flavoviridis*).

METHEMOGLOBIN — White snake venoms of *B. itapetiningae*, *C. d. terrificus* and one kind of white venom of *B. jararaca* did not transform hemoglobin to

TABLE 2 — Comparison of coagulant (CI), fibrinolytic (FI) and hemolytic (HI) indexes, of animal venoms.

Coagulant and Fibrinolytic indexes from Rosenfeld, Hampe & Kelen (30)
0 — absence of activity + clotting after 12 hours

VENOMS	C.I.	Corrected values	
		F.I.	H.I.
Apis mellifica	+	0,16	2,20
Naja naja	0	0,17	1,28
Vipera lebetina	+	0,24	1,26
Bungarus caeruleus	—	—	1,22
Vipera ammodytes montandoni	0	0,16	1,17
Vipera russellii	0	0,14	1,14
Bothrops alternatus	0,51	0,24	1,12
Bothrops jararaca (young snake)	3,92	0,50	1,12
Bothrops insularis	2,28	0,93	1,06
Crotalus durissus durissus	0,04	0,49	1,06
Lachesis muta muta	0,15	0,45	1,06
Bothrops atrox (Marajó island)	—	—	1,04
Bothrops fonsecai	1,62	0,21	1,04
Bothrops jararaca (*standard*)	*1,00*	*1,00*	*1,00*
Bothrops atrox	3,20	0,81	0,97
Bothrops neuwiedi	1,94	0,62	0,97
Echis carinatus	—	—	0,95
Bothrops atrox asper	3,18	0,45	0,93
Bothrops itapetiningae	0,31	0,64	0,92
Bothrops pradoi	—	—	0,91
Crotalus durissus terrificus (Ceará)	—	—	0,89
Trimeresurus flavoviridis	0	0,14	0,81
Bothrops jararacussu	0,03	0,24	0,52
Agkistrodon piscivorus	0	0,68	0,45
Crotalus durissus terrificus (yellow venom)	0,29	0,17	0,45
Crotalus durissus terrificus (white venom)	0,05	0,09	0,45
Bufo marinus	+	0,27	0,16
Crotalus durissus terrificus (Marajó island)	0,03	0,22	0,11
Bothrops cotiara	1,37	0,19	0,03
Micrurus frontalis	+	0,22	0
Tityus bahiensis	+	0,16	0
Tityus serrulatus	0	0,11	0
Androctonus australis	—	—	0
Lycosa erythrognata	+	0,16	0
Phoneutria fera	0	0,25	0
Loxosceles rufipes	—	—	0
Bothrops jararaca (white venom)	0,71	—	0

methemoglobin, while all yellow venoms had this activity that probably depends on the flavins existing in these venoms. This hypothesis may be reasonable since the yellow colour of *B. jararaca* venom is due to the presence of flavins as it has been demonstrated by Taborda and Taborda (34). And venoms of same species, when white, *i.e.*, lacking the flavin, did not transform hemoglobin, while the yellow ones did.

STABILITY OF HEMOLYTIC ACTIVITY — Storage of venom for a long time, practically does not change its hemolytic activity. There is only a potency decrease, without disappearence of any component, remaining all the characteristics of the hemolysis curve (fig. 2).

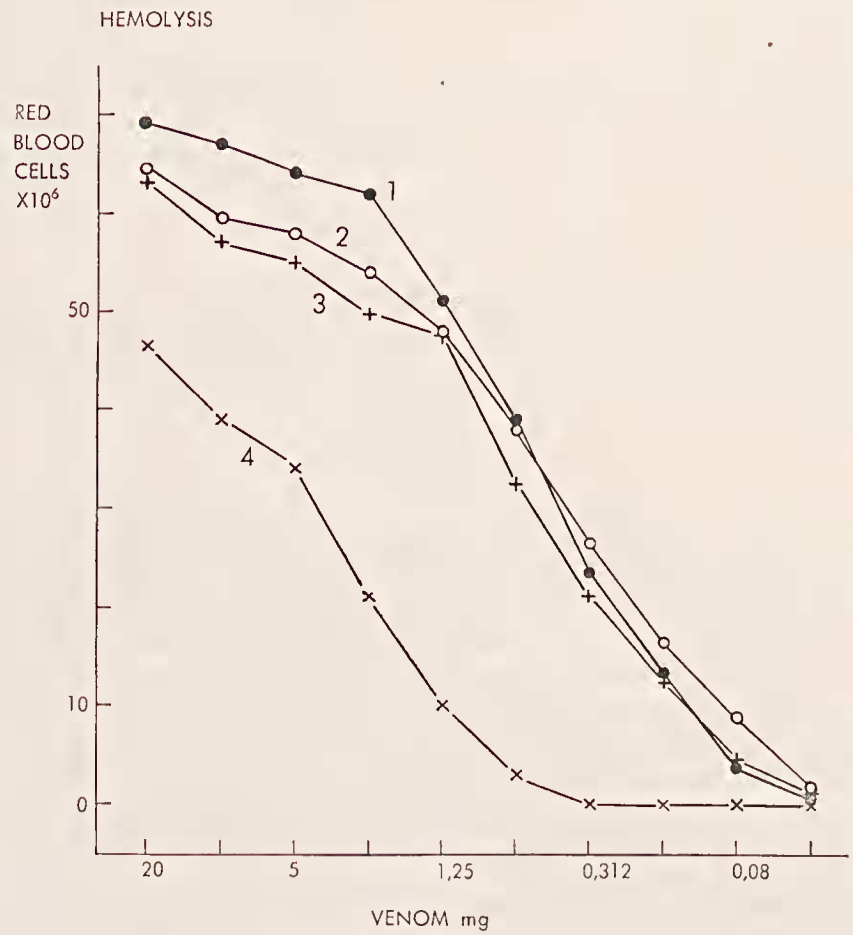

FIG. 2 — Influence of storage of *B. jararaca* venom on the hemolytic activity: Curve 1 — desiccated standard venom, 2 years old; curve 2 — Lyophilized venom, 3 months old; curve 3 — desiccated venom, 3 months old; curve 4 — desiccated venom, 14 years old.

SUMMARY

Hemolytic activity of venoms of 29 snakes, 3 spiders, 3 scorpions, 1 frog and bee was studied. Only the latter showed direct hemolytic activity (on washed red cells, without serum addition). The method utilized permitted a quantitative analysis of venoms which could be classified by their hemolytic index in relation to *B. jararaca* venom considered as standard. Constant quantities of venom (5 mg) and of sheep erythrocytes (100 x 10^6) were incubated in presence of horse serum. Hemolysis was colorimetrically determined by the liberated hemoglobin.

All snake venoms were hemolytic though venoms of *Micrurus frontalis* and *Bothrops cotiara* had been exceptions.

Spider venoms, *Lycosa erythrognata*, *Phoneutria fera*, and *Loxosceles rufipes* and scorpion venoms, *Tityus bahiensis*, *T. serrulatus* and *Androctonus australis* did not hemolyze red cells.

Determinations of hemolysis provoked by serial dilutions of one venom, starting from 20 mg, showed curves that permitted to group all venoms in 6 types. The different types of curve show clearly that there exist several hemolytic substances and differences in their number in each venom. Similarity in the curve of hemolytic activity was observed in venoms of different snake families as well as divergence among ophidians of the same genus.

All yellow venoms, hemolytic or not, had transformed hemoglobin to methemoglobin. White venoms whether hemolytic or not, did not have this activity, thus showing a direct correlation between yellow colour of venoms and methemoglobin formation.

RESUMO

Foi estudada a atividade hemolítica de 29 venenos de serpentes, 3 de aranhas, 3 de escorpiões, 1 de sapo e de abelha. Sòmente o último veneno mostrou capacidade hemolítica direta (sôbre glóbulos lavados sem a presença de sôro). O método usado permitiu uma análise quantitativa dos venenos que puderam ser classificados pelo seu índice hemolítico em relação a um veneno de *B. jararaca* tomado como padrão. Usou-se quantidades constantes de veneno (5 mg) e glóbulos de carneiro (100 x 10^6) incubados em presença de sôro de cavalo. A hemólise foi determinada colorimètricamente pela hemoglobina liberada.

Todos os venenos ofídicos foram hemolíticos, com exceção dos venenos de *Micrurus frontalis* e *Bothrops cotiara*.

Venenos de aranha, *Lycosa erythrognata*, *Phoneutria fera* e *Loxosceles rufipes*, e de escorpião, *Tityus bahiensis*, *T. serrulatus* e *Androctonus australis* não hemolisaram os glóbulos usados.

Determinações da hemólise provocada por uma série de diluições de um mesmo veneno, a partir de 20 mg, forneceram curvas que permitiram agrupar

os venenos em 6 tipos. Os diferentes tipos de curva evidenciam que pode haver várias substâncias hemolíticas e diferença de seu número de veneno para veneno. Foi observada semelhança da curva de atividade hemolítica entre venenos de serpentes de famílias diferentes, assim como diversidade entre ofídios do mesmo gênero.

Todos os venenos amarelos, hemolíticos ou não, transformaram a hemoglobina em metahemoglobina. Nenhum veneno branco, quer hemolítico quer não, teve esta propriedade, mostrando dêsse modo a correlação direta entre a côr amarela do veneno e formação de metahemoglobina.

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Venoms of *Bungarus caeruleus, Vipera russellii, Ecchis carinatus* and *Naja naja* were lyophilized and were kindly supplied by Dr. Deoras, of Haffkine Institute, Bombay, India.

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